THE DEPOSITION OF SKELETAL STRUCTURES IN THE CRUSTACEA. I. THE HISTOLOGY OF THE GASTROLITH SKELETAL TISSUE COMPLEX AND THE GASTROLITH IN THE CRAYFISH, ORCONECTES (CAMBARUS) VIRILIS HAGEN—DECAPODA[1]

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Little is known regarding the basic mechanisms by which certain cells and tissues participate in synthesis and calcification of organic matrices. The gastrolith discs and the branchial exoskeleton provide particularly useful experimental material in the Crustacea for such studies. Attention, in this paper, will be confined to the histological study of the gastrolith discs of the fresh-water crayfish, *Orconectes virilis* Hagen.

The paired gastrolith discs are composed of the cuticular lining of the stomach, the thickened gastric epidermis, and the underlying sub-epidermal connective tissue. They are located in the anterior lateral walls of the cardiac stomach of the crayfish. Their usefulness as sites of activity for the study of cellular processes involved in the synthesis and calcification of organic matrices is evident when it is realized that the epidermis of these modified portions of the stomach wall becomes competent to synthesize and calcify the gastrolith matrix preceding molt. This activity culminates at the end of the premolt period in the formation of hard calcified disc-shaped gastroliths which lie in a sac or pouch now formed between the epidermis and cuticular lining of the stomach. At the same time gastrolith formation occurs, the epidermis of the exoskeleton is participating in resorption of mineral and organic constituents. At the molt, the fully formed gastroliths are shed with the old stomach lining into the stomach. Following molt, these gastroliths are gradually broken down and resorbed. Some of their mineral constituents are resorbed by the gastrolith epidermis and hepatopancreatic epithelium (Travis, in preparation). These mineral constituents are conveyed by the blood to the epidermis underlying the exoskeleton of other areas and are re-used in synthesis and calcification of their organic matrices.

While the description of the presence of gastroliths before molt and their gradual disappearance following molt has been given by a number of authors (Réaumur, 1712; Chantran, 1874a, 1874b; Braun, 1875; Huxley, 1879; Herrick, 1895; Irvine and Woodhead, 1889; Husson, 1952; and others), only certain aspects of the histology of the gastrolith discs have been described by Braun (1875) for the European crayfish, *Astacus fluviatilis,* and by Herrick (1895) for the American lobster, *Homarus americanus.* The histological changes have neither been described with reference to stages of the molting cycle nor to the synthesis of the non-calcified

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skeletal components of the gastrolith discs, and only with brief reference to the synthesis and calcification of the gastrolith itself. Accordingly, it is the aim of the present paper to deal with histological changes which are associated with the formation of non-calcified skeletal components of the gastrolith discs and those involved in the synthesis and calcification of the gastrolith itself.

Materials and Methods

Animals. Since the duration of each stage of the molting cycle, the length of the intermolt periods, and frequency of molts for each size group have not been established for either field or laboratory *Orconectes virilis,* only males ranging in size from 40–49 mm. carapace length and molting during July and August were used in these studies. All animals were previously collected from the Cambridge Reservoir and maintained in the laboratory in concrete tanks containing rocks and soapstone slabs placed over the bottom to provide secluded areas for crayfish retreat and basking. A gently flowing stream of water, ranging in temperature from 21.8–25.0° C., was maintained at a level of approximately 2.5 inches in the tanks.

Stages of the molting cycle. Stages of the molting cycle were determined according to Drach (1939, 1944). This method is based primarily on morphological features of the exoskeleton, and clearly delimits four major stages—A, B, C, D—each of these being divisible into a number of substages. *Postmolt,* consisting of Stages A, B, early, and middle C, is characterized by progressive hardening of the preexuvial layers; formation, progressive thickening and hardening of the endocuticle; the beginning of feeding; and formation and progressive thickening of the membranous layer. The *intermolt condition* (Stage C_4) is characterized by the completion of all components of the exoskeleton—the epicuticle, exocuticle, endocuticle, and membranous layer. At this stage, one of comparative "rest" or "stability," the completed membranous layer with the adhering epidermis can be stripped from the remaining portion of the exoskeleton. *Premolt,* consisting of early, middle, and late Stage D, is characterized by a series of integumentary transformations which occur preparatory for the ensuing molt. The major portions of the old exoskeleton, both organic matrix and mineral salts, are resorbed; the new preexuvial layers, consisting of the epicuticle and exocuticle, are deposited under the old; the animals cease to feed; and the termination of the period is marked by the molt. Stage D_0 is a new stage, previously referred to by Drach in connection with the shrimp molting cycle (Comments at Harvard, 1957). Stage D_0 in the crayfish is a stage in the molting cycle which cannot be distinguished from Stage C_4 by external features of the exoskeleton. The membranous layer with the adhering epidermis can be stripped from the exoskeleton in both stages but in Stage D_0, gastrolith deposition has begun, the gastroliths in this case being thin plate-like structures.

Histological methods. Three to five animals were killed for each specific stage of the molting cycle. The gastrolith discs were quickly removed, placed on a glass slide and straightened in a drop of fixative before they were transferred to bottles containing larger volumes of fixative. Although the author has used various fixatives for general histological studies of crustacean material (1951, 1955, 1957),

Bouin's fixative containing 1% calcium chloride (anhydrous) was used in these studies and has proved to be the best fixative. Fixation was carried out for at least a 24-hour period, followed by washing in 70% ethyl alcohol for the same period, dehydration in 80%, 95%, 97% ethyl alcohol for 30 minutes each, two changes of 100% ethyl alcohol for 15 minutes each and infiltration according to Peterfi's Celloidin-paraffin Method (Pantin, 1948). Sections were cut at 6 μ, deparaffinized, coated with 0.5% celloidin for one minute, allowed to dry and stained with Mallory's triple stain and Ehrlich's haematoxylin and eosin for general histological studies.

Observations and Discussion

The intermolt condition—Stage C_4

Intermolt is marked not only by the completion of the calcified skeletal components and the membranous layer of the rigid exoskeleton, but by the completion of the non-calcified skeletal components of the gastrolith discs. The epicuticle of

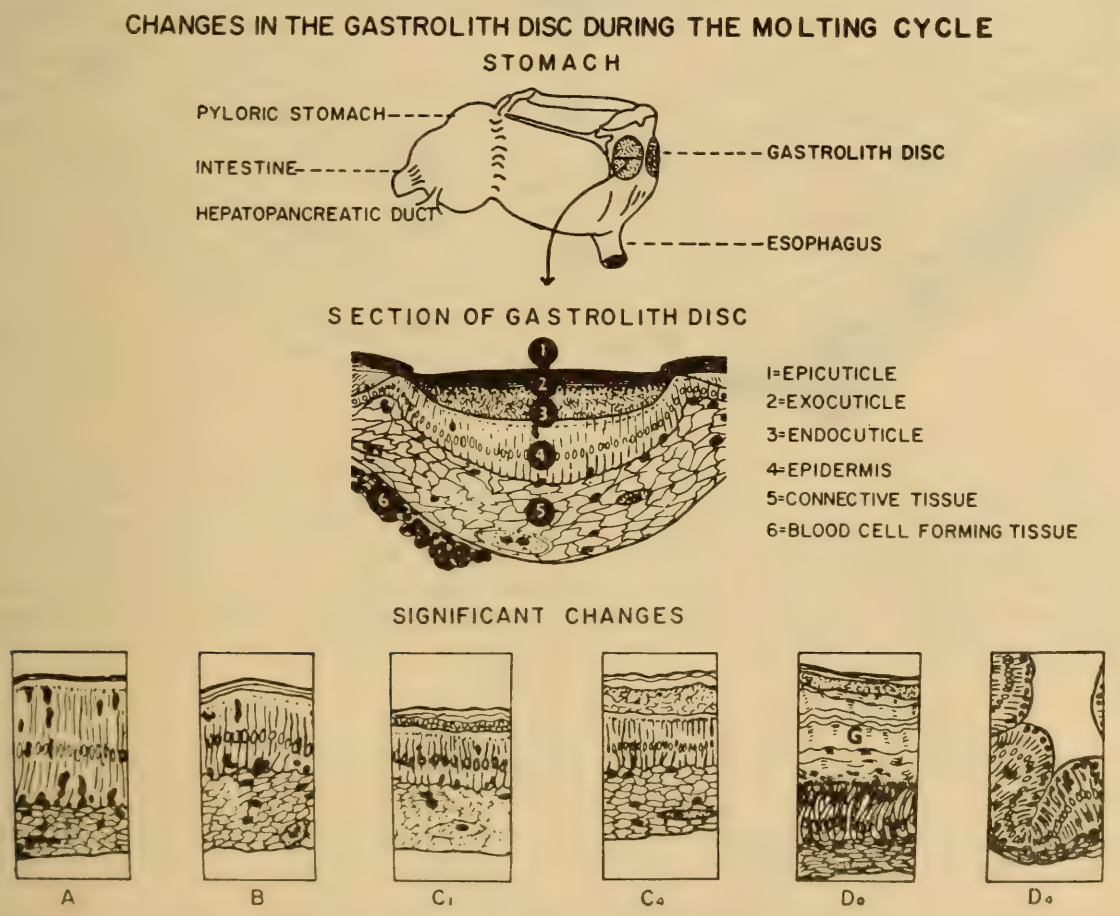


Figure 1. Diagram showing the histological changes which occur in the gastrolith disc during significant stages of the molting cycle. Enlarged section of the gastrolith disc is from an intermolt animal (Stage C_4), in which all skeletal components of the disc are completed, more clearly shown in Figure 2. Note significant changes in the development of cuticular components, epidermal height, connective tissue thickness, abundance of reserve cells (represented as black oval bodies). In Stage D_0, note gastrolith (G) forming between epidermis and cuticular components of the disc, and in Stage D_4, note hypertrophy and folding of the epidermis after it has retracted from the fully formed gastrolith and undergone a spurt of growth.

these latter components, adjacent to the lumen of the stomach, constitutes one surface of each gastrolith disc, while the basement membrane of the sub-epidermal connective tissue, adjacent to the hemocoel, constitutes the other surface of each disc (Figs. 1, 2, 4). Although the gastrolith discs, a few millimeters in diameter, are modified portions of the stomach wall, they can be distinguished from the remainder of the stomach wall in Stage C_4 by distinct differences in the structure and composition of their cuticular components and by the nature of their epidermis.

The completed skeletal components of the gastrolith discs of an intermolt animal show three major differentiated layers in contrast to the four observed in

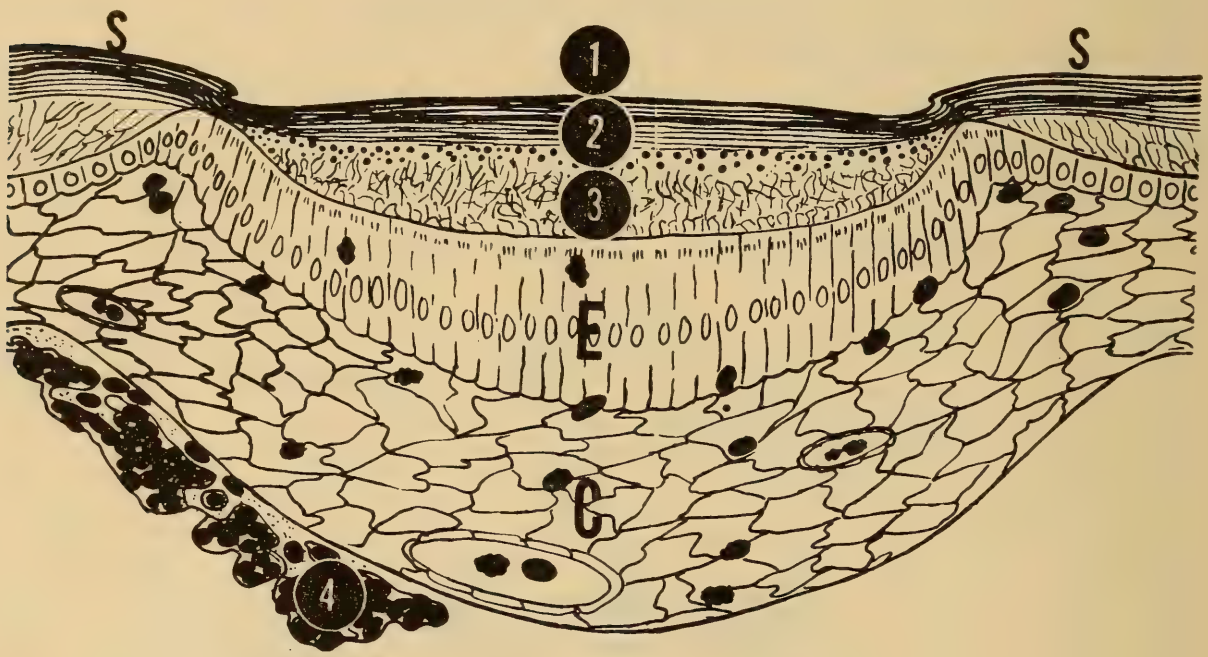


FIGURE 2. Enlarged diagram of the gastrolith disc of an intermolt animal (Stage C_4). Note the three differentiated skeletal layers—(1) epicuticle, (2) laminated exocuticle, (3) endocuticle with characteristic, chemically complex granules which mark the gastrolith disc histologically from the ordinary stomach lining (S). Note the tissue complex of gastrolith disc, which consists of a single layer of columnar epidermal cells (E), the sub-epidermal connective tissue (C), composed of cells of Leydig, and the transient reserve cells (represented as black oval bodies). Also note the blood cell-forming gland (4) and blood vessels in the connective tissue.

the more rigid branchial exoskeleton. Beginning with the lining of the stomach (Figs. 1, 2, 4), these are:

1. *The epicuticle.* This is a very thin non-calcified component, approximately 3–4 μ in thickness and composed of a glyco-lipoprotein complex (Travis, in preparation). It is neither laminated nor crossed by pore canals or tegumental ducts. Elaboration and hardening of the epicuticle begins before molt (Stage D_4) and is completed by Stage C_1. This epicuticle differs from that of the branchial exoskeleton in being thinner and not being calcified or crossed by tegumental ducts. It stains red with Mallory's.

2. *The exocuticle.* This is a laminated component of approximately 8–12 μ in thickness and is also composed of a glyco-lipoprotein complex (Travis, in prepara-

tion). Deposition does not begin until late Stage A and is completed in Stage B. In contrast to the exocuticle of the branchial skeleton the gastrolith disc exocuticle is thinner, non-calcified, lacks pore canals and tegumental ducts, and is formed during a different stage of the molting cycle. The outer zone stains light blue with Mallory's while the inner stains dark blue, with a reddish haze at the junction of the exo- and endocuticle.

3. *The endocuticle.* This is the thickest of the three layers. It reaches a maximum thickness of 15–25 μ in Stage C_4 and stains very light blue with Mallory's. This layer differs from the same layer of the branchial exoskeleton in that it is neither laminated nor crossed by pore canals or tegumental ducts. The endocuticle begins to be deposited in late Stage B and is completed by Stage C_4. It appears as a loose fibrous meshwork and is marked by the presence of round structureless granules (Figs. 1, 2, 4), which also stain red with Mallory's. These granules reach a size of 2.5–3.0 μ, the largest being located immediately under the exocuticle. These are chemically complex granules which contain a glycoprotein complex, a mixed lipid complex, glycogen at certain stages of the molting cycle, and calcium (Travis, in preparation). Such granules are not observed in the endocuticle of the ordinary foregut lining.

The tissue underlying the skeletal components is composed of a single layer of columnar epidermal cells which lie in immediate contact with the endocuticle (Figs. 1, 2, 4). These columnar epidermal cells are approximately 40 μ in height with nuclei of about 5×13 μ. The epidermal layer of the remaining portion of the stomach is a layer of cuboidal or low columnar cells.

The sub-epidermal connective tissue is very similar to that observed in the branchial integument. It is predominantly composed of the loose "spongy" type of connective tissue cells known as "cells of Leydig" (Figs. 1, 2, 4). A number of transient cells, called "protein cells" by Cuénot (1891, 1893), blood corpuscles by Hardy (1892), "reserve cells" (Travis, 1951, 1955, 1957), "lipoprotein cells" (Sewell, 1955), are also evident during the intermolt condition. These transient or reserve cells contain both acidophilic and basophilic granules when stained with haematoxylin and eosin and are usually fuchsinophilic with Mallory's. The granules vary in size, as observed with both the light and electron microscope (Travis and Chapman, II), from less than one micron to around four microns, and have the same histochemical composition as observed in the granules of the endocuticle (Travis, in preparation). The fine-structure studies indicate that the mature reserve cells contain large refractile granules which show variable densities and double membranes, while the immature cells are smaller, may contain clearer non-refractile cytoplasm and smaller granules of variable densities. These cells, among other functions, appear to be intimately involved in transport and release of reserves for synthesis and elaboration of skeletal elements (a more detailed discussion of these cells will be given in a subsequent paper of this series). In addition to these cellular elements, the sub-epidermal connective tissue is well penetrated by branches of the antennary arteries and blood sinuses. Also present in the connective tissue is a conspicuous blood cell-forming gland (Figs. 1, 2), in which stages of amoebocyte or reserve cell development may be seen. This blood cell-forming gland has also been observed by Małaczyńska-Suchcitz and Hryniewiecka (1958). Delimiting the connective tissue from the hemocoel is a basement membrane.

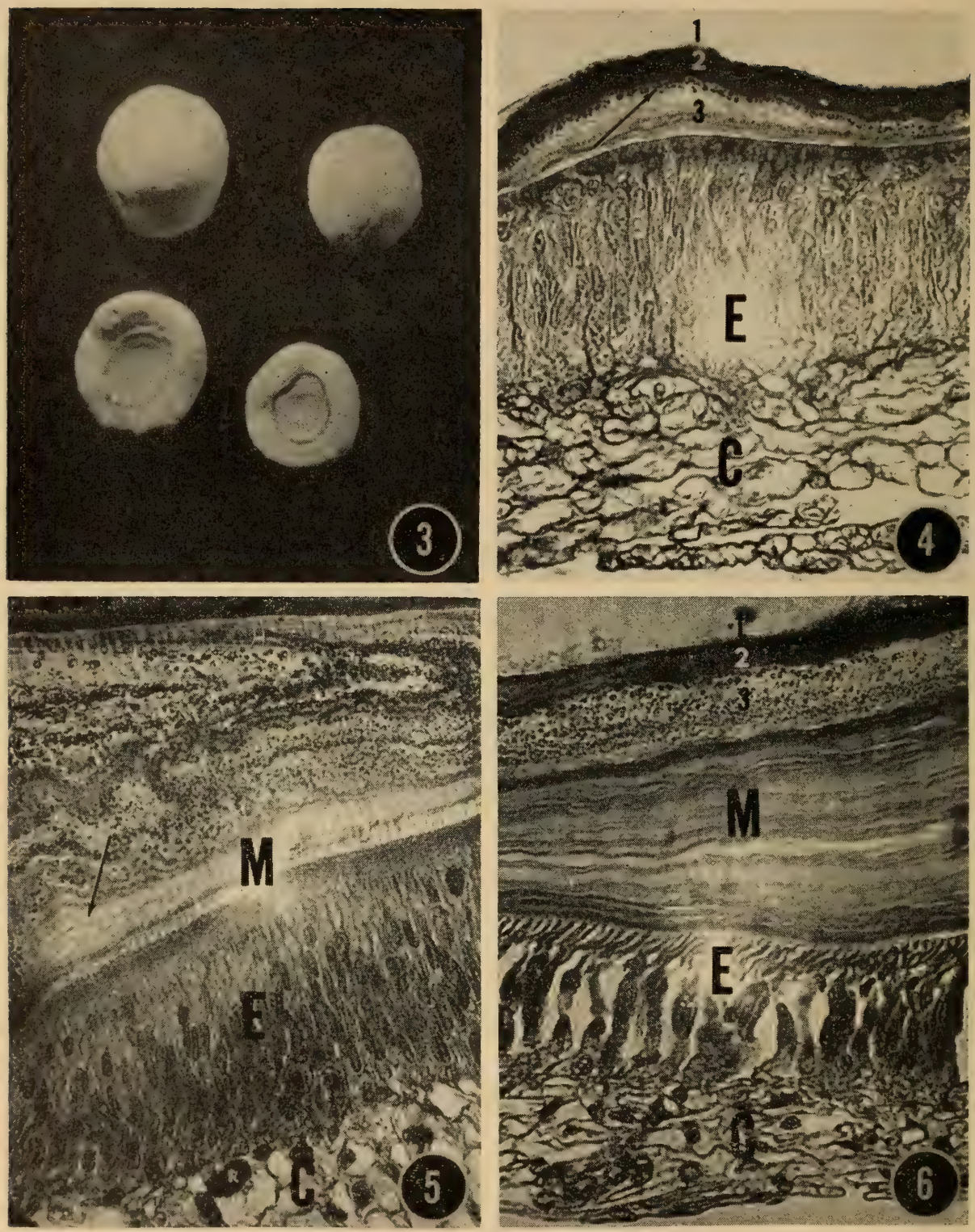


FIGURE 3. Two pairs of gastroliths removed from the gastrolith pouches. The exposed convex surface of the upper pair would lie *in situ* in immediate contact with the epidermis. The concave surface of the lower pair would lie in immediate contact with the stomach lining. 3 ×.

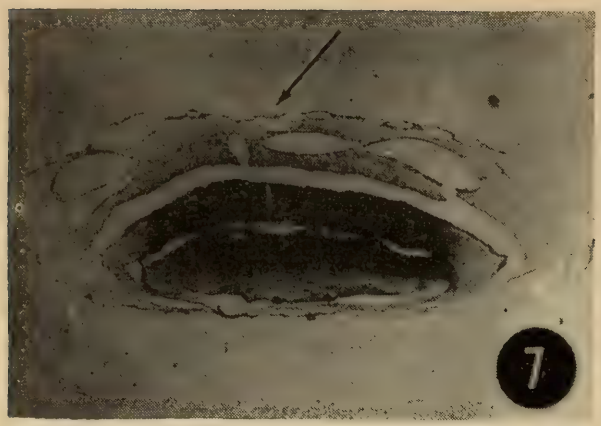
FIGURE 4. Section of the gastrolith disc skeletal-tissue complex (Stage C_4). The epicuticle (1), the exocuticle (2), the endocuticle (3) containing its characteristic chemically complex granules (arrow), the epidermis (E) and the connective tissue (C). 1800 ×.

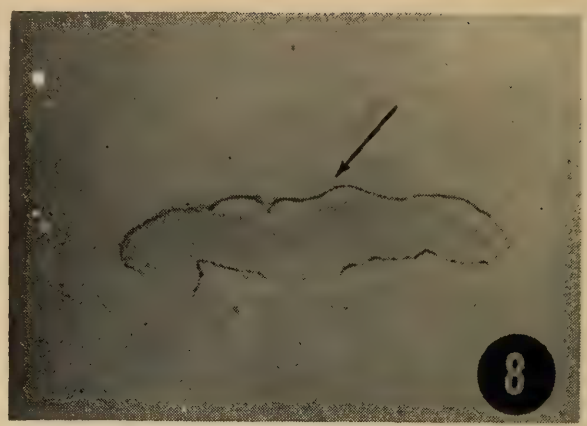
Premolt-Stage D_0 through D_4

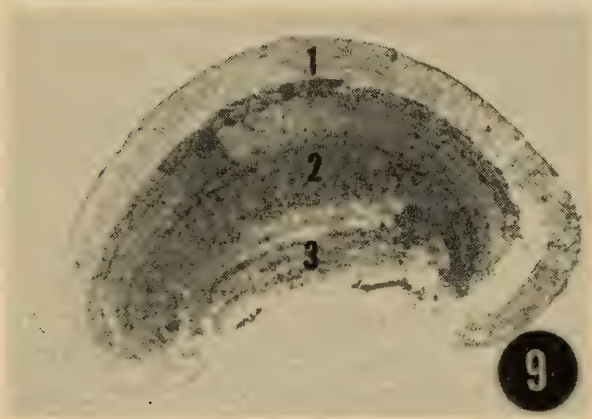
During this period of the molting cycle, marked histological changes, associated with gastrolith deposition, occur in the gastrolith disc tissues. In Stage D_0, the epidermis (with cells of about 75 μ in height and bearing nuclei of around 5 × 12 μ) becomes somewhat invaginated. In section, this is evident at the very edge of the gastrolith disc (Fig. 5). These active epidermal cells shortly become branched and attenuated at their apical ends and many intercellular spaces are then apparent between the cells (Figs. 1, 6). Their attenuated apices lie in immediate contact with the forming matrix of the gastrolith. At the ultrastructure level, it is evident that the attenuated apices observed with the light microscope also bear innumerable microvillar equivalents (Travis and Chapman, III). From the microvillar equivalents both organic and mineral material are secreted, some microvillar equivalents secreting granular material into the forming amorphous matrix while others secrete distinctly beaded-fibrous material. The secreted organic components, not observable with the light microscope, undergo polymerization to form the fibrous lamellae (Travis and Chapman, II, III) clearly observed in the matrix of the gastrolith with the light microscope (Figs. 6, 7, 8). Evidence at the ultrastructure level also reveals that calcium transport and deposition appear to occur by two different means (Travis and Chapman, III). The granular and beaded-fibrous components secreted from the microvillar equivalents undergo a change. The former gives rise almost immediately to small crystals of calcite ranging in size from 440–1060 Å, while the beaded components of the latter eventually give rise to the same size crystals, presumably of calcite. Crystal formation from the beaded-fibrous components occurs simultaneously with polymerization changes associated with the formation of the fibrous lamellae of the matrix (Travis and Chapman, III). These small crystals, which form in the matrix of the young gastrolith (Stage D_0) and which are not observed with the light microscope, grow to a size of 7 μ or more in diameter and are clearly observed in ground sections of the completed gastrolith (Figs. 12, 13). These ground sections of the completed gastrolith (Stage D_4) also reveal that there is a shift in the mode of crystal deposition as the youngest and last layers of the fully completed gastrolith are deposited. In this case, crystals are deposited in such a fashion that rows of crystal prisms or rods (Fig. 10) are laid down parallel to the longitudinal axis of the epidermal cells and perpendicular to the parallel lamellae of the gastrolith (Fig. 9). The thickness of the younger layers of the gastrolith is constituted of these crystal prisms or rods, with one crystal stacked vertically above another. This thickness is 490–500 μ as compared to the older portions of the gastrolith (Figs. 9, 11, 12, 13), which constitute a thick-

FIGURE 5. Section through the extreme edge of the developing gastrolith (Stage D_0). Note that the developing matrix (M) is filled with granules similar in appearance and chemical composition to those observed in the completed endocuticle. Also note beginning appearance of fibrous lamellae (arrow). Epidermis is denoted by (E), connective tissue by (C) and reserve cells (R). 1800 ×.

FIGURE 6. Section through a more central portion of the developing gastrolith (Stage D_0). Note the distinct fibrous lamellae of the gastrolith matrix (M) which result from post-secretion polymerization changes, the attenuated apices of the epidermal cells (E) and the intercellular spaces between the cells, and the connective tissue (C). Also note the three differentiated skeletal layers of the gastrolith disc—(1) epicuticle, (2) exocuticle, (3) endocuticle with characteristic granules. 1800 ×.







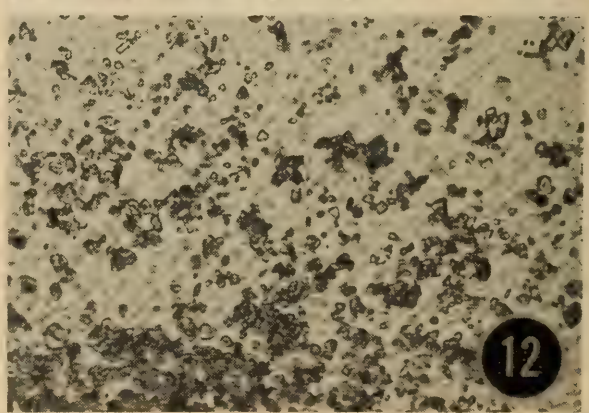


FIGURE 7. A section through the organic matrix of a completed gastrolith (Stage D_4) showing its fibrous lamellate nature. Fixed and decalcified in ordinary Bouin's and stained with toluidine blue. The more convex surface (arrow) would lie *in situ* in immediate contact with the epidermis while the opposite surface would lie in contact with the stomach lining. 16 ×.

FIGURE 8. A similar section through the organic matrix of the early developing gastrolith (Stage D_0), showing its fine fibrous lamellate nature. Arrow indicates surface (younger layers) which would lie *in situ* in contact with the epidermis. 21 ×.

ness of 2500–3000 μ and which show lamellae and crystals oriented at right angles to the prisms. Preliminary x-ray diffraction studies indicate that the main inorganic crystals in both the youngest and oldest layers of the gastrolith are calcium carbonate deposited as calcite.

The sub-epidermal connective tissue becomes greatly compressed or stretched during this stage of the molting cycle. This is largely due to the size of the developing gastrolith which is plate-like in nature (Stage D_0) and has attained a thickness at this time of about 300 μ. By middle Stage D, the gastrolith has increased to a thickness of approximately 1 mm. and by the end of D_4, a thickness of approximately 3–4 mm. is achieved in animals of 40–49 mm. carapace length. Reserve cells remain numerous in the connective tissue and epidermis during the entire premolt period, being heavily concentrated in the apices of the epidermal cells, and achieve their greatest abundance in late Stage D (D_4). During this latter stage the epidermis has completed the deposition of the gastrolith and retracts from it, undergoes rapid growth, accompanied by much folding, and achieves its greatest height (242 μ) and nuclear size of 9 × 14 μ (Fig. 14). It now begins to elaborate a new epicuticle which is the only skeletal component of the gastrolith disc deposited before molt. In areas of the stomach wall on each side of the gastrolith discs both pre-exuvial layers, epi- and exocuticle, have been deposited. When synthesis of the gastrolith and new epicuticle are achieved by the gastrolith disc epidermis, correlated with the many tasks the epidermis performs in other skeletal areas, molting ensues and the old stomach lining and the completed gastroliths (Fig. 3) are released into the stomach.

Postmolt-Stage A, B and C

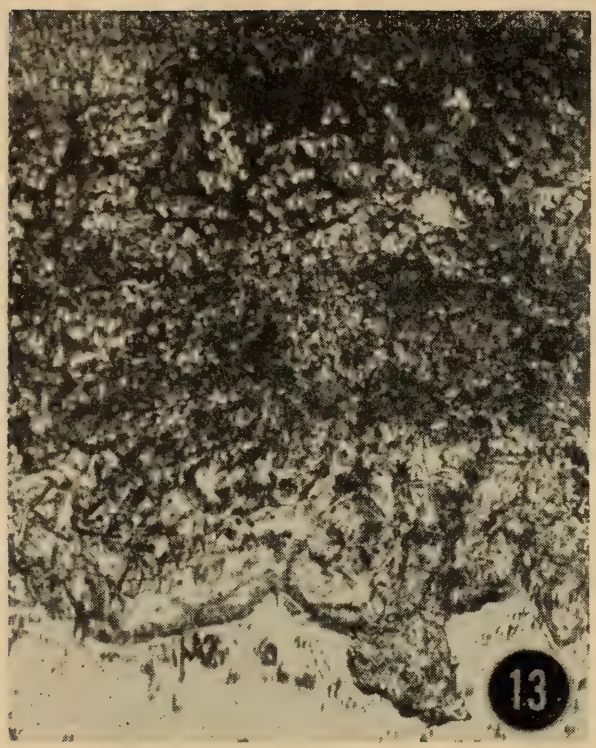
Stage A. The tissue complex of the gastrolith disc remains greatly hypertrophied. The epidermis, in contrast to the folded appearance in Stage D_4, is a flat-surfaced tissue, composed of rows of columnar cells, which maintain a height of approximately 182 μ and an average nuclear size the same as that of a D_4 animal (Fig. 15). The reserve cells remain abundant in both the epidermis and connective tissue, with the greatest number being observed in the apices of the epidermal cells. The only skeletal component observed in the gastrolith disc early in Stage A

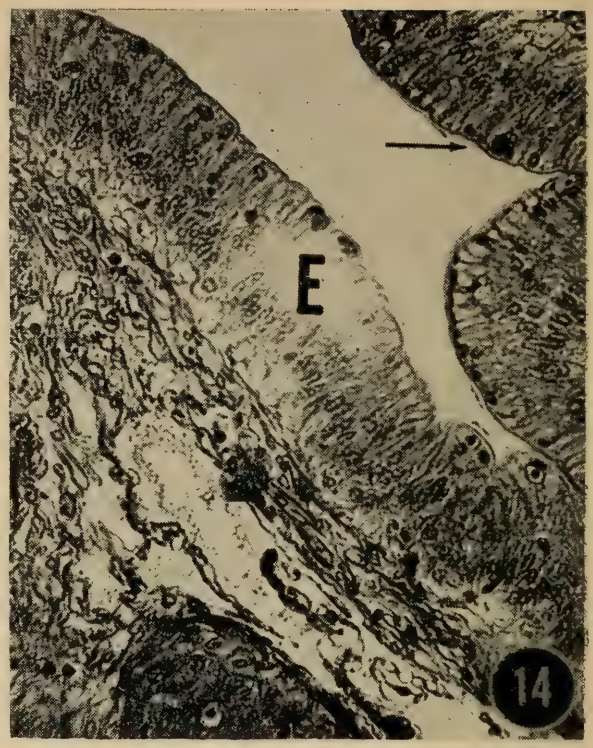
FIGURE 9. A ground section of a fully developed gastrolith (Stage D_4). The youngest portion (1) would be in contact with the epidermis, and consists of crystal prisms or rods, also indicated in Figure 10, which are laid down parallel to the longitudinal axis of the epidermal cells and perpendicular to the parallel lamellae of the gastrolith. Region (2), also represented in Figure 11, is an older portion of the gastrolith showing the parallel lamellae with crystals oriented at right angles to the prisms of region (1). Region (3) represents the oldest layers of the gastrolith, originally corresponding to Stage D_0, and would lie in contact with the skeletal layers of the gastrolith disc which constitute part of the stomach lining, also represented in Figures 12 and 13. 8.5 ×.

FIGURE 10. Enlarged portion of region (1) from a ground section of a completed gastrolith, depicted in Figure 9. Note crystal prisms or rods of calcite. 400 ×.

FIGURE 11. Enlarged portion of region (2) from a ground section of a completed gastrolith depicted in Figure 9. Note that crystals of calcite in the lamellae (arrows) lie at right angles to the crystal prisms of region (1). 400 ×.

FIGURES 12, 13. Enlarged portions of region (2) from a ground section of a completed gastrolith depicted in Figure 9. Note same arrangement of crystals of calcite—7–70 μ in diameter—as observed in Figure 11. 400 ×.





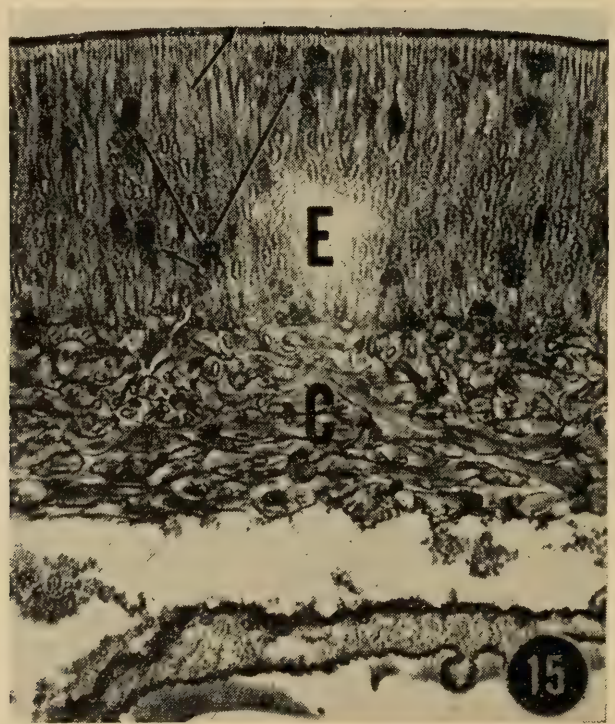


FIGURE 14. Section of gastrolith disc showing greatly hypertrophied and folded epidermis (E), following retraction and growth of this tissue after gastrolith deposition is complete (Stage D_4). Note beginning of new epicuticle formation (arrow) and numerous dark staining reserve cells in the apices of the epidermal cells. 900 ×.

FIGURE 15. Section of gastrolith disc immediately following molt (Stage A). Note the greatly hypertrophied flat-surfaced epidermal cells (E), the epicuticle (arrow), the only skeletal component evident in early Stage A, and the numerous dark staining reserve cells (arrow-R), and connective tissue (C). 900 ×.

is the epicuticle, partly formed and hardened previous to molt and having a thickness of only 1.6–2.0 μ. Curiously enough, the exocuticle does not apparently begin to be deposited until late Stage A and is completed in Stage B, achieving a thickness of approximately 8–12 μ. In the areas of the stomach wall on each side of the gastrolith discs, the exocuticle is completed and endocuticle formation has already begun.

Stage B. This stage of gastrolith disc formation is marked by the completion of the synthesis and elaboration of the exocuticle. It is both fibrous and laminated in nature. This latter aspect is not clearly evident at the microscopic level, but is distinctly apparent in electron micrographs (Travis and Chapman, II). The synthesis and elaboration of the endocuticle also begins during the latter part of this stage but is not completed until late Stage C.

There is a slight decrease in epidermal cell height (112 μ) and nuclear size (5.1 × 11.5 μ) and an increase in thickness of the connective tissue when compared to the situation observed in Stage A. During this stage the reserve cells are still quite abundant.

Stage C. Early Stage C (C_1) and middle Stage C (C_2) are marked by the reduction in epidermal height (about 80 μ), a slight increase in nuclear size (5 × 13 μ), an increase in thickness of the sub-epidermal connective tissue, a gradual reduction in the number of reserve cells, and the continued synthesis of the endocuticle, previously described under the intermolt condition. It is during Stage C_1 that the interesting granules, observed in the gastrolith disc endocuticle, become evident at the ultrastructure level, although not becoming distinctly apparent at the light microscope level until Stage C_4. In the tissue complex there is little difference between the early and middle Stage C condition and that observed in the intermolt animal. At the end of Stage C when synthesis of the gastrolith disc skeletal components is complete, the animal has again reached an "intermolt" condition (C_4) from which the entire cycle repeats itself. Further information on the development of the non-calcified skeletal components of the gastrolith disc and of the calcified gastrolith itself will appear in subsequent papers of this series.

Summary

1. The gastrolith discs of the fresh-water crayfish, *Orconectes virilis* Hagen, are located in the anterior lateral walls of the cardiac stomach and are themselves modified portions of the stomach wall.

2. Histologically, the cuticular surface of the completed gastrolith disc (Stage C_4), which forms part of the lining of the stomach, is composed of three differentiated layers: a thin non-calcified epicuticle, formed before molt; a thicker non-calcified exocuticle, laminated in nature, but neither crossed by pore canals, nor tegumental ducts, formed following molt and completed during Stage B; and the endocuticle, the thickest of the three layers, characterized by neither being laminated nor crossed by pore canals and possessing round granules without structure, embedded in a loose fibrous meshwork. The largest of these granules are located in the outer layers of the endocuticle very close to the exocuticle. The tissue complex of the completed gastrolith disc is composed of a single layer of columnar epidermal cells, a loose "spongy" type of sub-epidermal connective tissue, composed of cells of Leydig and a number of the transient reserve cells which are also observed in the

epidermis. The tissue complex is well supplied by branches of the antennary arteries and has a large blood-cell-forming gland.

3. Marked histological changes are observed during Stage D when premolting processes, associated with gastrolith deposition, are set into motion. The epidermis increases greatly in height, invaginates, and begins to elaborate the matrix of the gastrolith which is filled at its lateral edges with granules like those observed in the C_4 endocuticle. At this same time the active epidermal cells take on a branched attenuated appearance at their apical ends, and many intercellular spaces become apparent between the cells. From Stage D_0 through most of Stage D_4 continued synthesis, elaboration, and calcification of the gastrolith matrix occur, its thickness increasing from around 300 μ (D_0) to 3–4 mm. (D_4). Concomitant with these changes is an increase in the number of reserve cells in both epidermis and connective tissue and a stretching, and thus compression, of the sub-epidermal connective tissues, the latter process being correlated with the increasing size of the forming gastrolith. Near the end of Stage D (D_4), the epidermis has completed its task of depositing the gastrolith, retracts from it, undergoes rapid growth accompanied by much folding and achieves its greatest height. It then begins to elaborate the epicuticle, the only pre-exuvial skeletal component deposited in this site. When this task is achieved by the epidermis, correlated with the many tasks it performs in other skeletal areas, molting ensues and the old stomach lining and the formed gastroliths are released into the stomach.

4. Postmolt histological changes in the gastrolith disc are associated with continued synthesis and elaboration of cuticular components. The "intermolt condition" is achieved in Stage C_4, when these synthetic tasks are complete, and from this stage the entire histological cycle repeats itself.

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